Comparative Qualitative Analysis of Osteogenic Precursor Cells Obtained from Human Periosteum, Cancellous Bone and Dental Pulp Tissue

Synopsis

Submitted by

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Abstract:

Background: Stem cells derived from intraoral sources have high utility in craniofacial bone regeneration. There are various sources of stem cells from the oral cavity with osteogenic potential. There have not been enough studies to assess or compare the osteogenic potential of stem cells from intraoral sources.

Aim: in vitro comparision of cell proliferation, osteoblastic activity and mineralization potential of three intraoral sources of stem cells – periosteum, alveolar bone and dental pulp.

Methodology: Periosteum alveolar bone and dental pulp will be harvested from consenting healthy individuals during routine oral surgical procedures. Periosteum derived stem cell (PDSC), Alveolar bone derived stem cell (ABSC) and Dental pulp derived stem cell (DPSC) will be isolated and cultured from the sources and compared for cell proliferation (MTT assay), osteoblastic activity (ALP activity) and mineralization potential (Alizarin red S staining) at day 7, 14, 21 and 28.

Results: All observations will be analysed using standard statistical tools to determine the quantitatively and qualitatively superior intra-oral source of stem cell for osteogenic regeneration.

Key words: - Periosteum derived stem cell, Alveolar bone derived stem cell, Dental pulp derived stem cell, Stem cells, osteogenic precursor cells

Introduction:

Origin of research problem

Craniofacial surgeons very often face clinical situations in which bone regeneration is required. Bone loss caused by various diseases (congenital or degenerative) trauma, surgical procedures is a problem for functionality and is having an ever increasing social impact. Current surgical techniques have used, in various combinations, autogenous, allogenic and prosthetic materials to achieve bone reconstruction. In the past the "gold standard" bone graft material was autogenous bone. An autograft is limited by several problems including limited supply, additional surgical time, donor site morbidity, long recovery period. Allografts have also beset with multitude of concerns, chief among which include infection, immunologic rejection, graft-versus-host disease and pathogen transmission. Alternative materials like metal alloys, glass, plaster of paris, polymethylmethacrylate, collagen and other biodegradable scaffolds have been used to assist in bone reconstruction. Tissue engineering by autogenous cell transplant is one of the most promising techniques currently being developed for bone regeneration. The aim of tissue engineering is the regeneration of tissues through the combined use of biomaterials and biologic mediators in order to provide new tools for regenerative medicine.

This process consists of three necessary elements (a) cells with osteogenic potential (b) scaffold (c) osteogenic growth factors. Several different types of cells have been used in tissue-engineered bone formation, including bone marrow stem cells, embryonic stem cells, periosteal cells, adipose cells, dental pulp stem cells, cells derived directly from bone and many other sources. In order to capitalize on the ability of the osteogenic precursor cells to induce bone formation in vivo, it is necessary to investigate the quantity and quality of these precursor cells provided by these sources.

Aim

To compare the quantity and quality of osteogenic precursor cells obtained from three sources in the oral cavity – periosteum, cancellous bone and dental pulp.

Interdisciplinary relevance

Langer and Vacant define tissue engineering as "an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain or improve tissue function". An important step in bone tissue engineering is the selection and manipulation of osteogenic cells. The culture and isolation of osteogenic precursor cells, their quantification and analysis for their viability and osteogenic activity involves a combined effort of clinical, laboratory and biomedical personnel with knowledge and skills in this field.

Review of research and development in the subject

a. Shi-Jiang Zhu et al. investigated the in-vivo potential of cultured bone marrow mesenchymal stem cells, cultured alveolar bone cells and cultured periosteal stem cells to form bone. Cells were isolated from dogs, expanded in vitro, mixed with autologous fibrin glue and BMP-2 and then injected into the subcutaneous space on the dorsum of nude mice. Bone formation was evaluated after 12 weeks. Their results showed that periosteal cells were the best choice for enhancing bone formation in tissue engineering of bone regeneration[1].

b. Y. Zhou et al. studied the effects of human platelet-rich-plasma (hPRP) on the proliferation and osteogenic differentiation of human adipose-derived stromal cells (hADSCs). hADSCs were isolated from liposuction tissues of healthy patients and hPRP was obtained by traditional two step centrifugation method. MTT analysis, alkaline phosphatase activity and mineralization activity were used to evaluate the effects of different concentrations of hPRP on cell proliferation and osteogenic differentiation in vitro[2].

c. Patrick H. Warnke et al. aimed to test the ability of four collagen membranes to support and promote the proliferation of human periosteal cells. Cell vitality was assessed by fluorescin diacetate (FDA) and propidium iodide staining, biocompatibility with LDH and BrdU, MTT, WST tests and scanning electron microscopy. It was concluded that collagen membranes can be used as scaffolds for cultivation of periosteum layers with a view to creating cortical bone using tissue engineering methods[3].

d. C. Ittichaisri et al. compared the proliferation and growth of human osteoblast-like cells on seven different biomaterials in vitro. Cell proliferation and colonization were analyzed by scanning electron microscopy and EZ4U-test. The study human osteoblast-like cells appear to be sensitive to substrate composition and topography and the cells cultivated on Ethisorb (synthetic biodegradable polymer) showed the highest proliferation rate[4].

e. Chang and Tate reviewed the unique features of periosteum-derived cells, particularly in light of translational therapies and engineering solutions. Periosteum tissue can be removed from many convenient locations and periosteal cells proliferate much faster than most MSCs while retaining their ability to differentiate. They remain viable after prolonged subcultivation and cryopreservation, do not exhibit contact inhibition, and continue to proliferate into high densities[5].

f. Riccardo d'Aquino et al. conducted a study where they used a biocomplex constructed from dental pulp stem-progenitor cells (DPCs) and a collagen sponge scaffold for bone tissue repair in patients requiring extraction of mandibular third molars. DPCs were isolated from extracted maxillary third molars and seeded onto a collagen sponge and the obtained biocomplex was used to fill the injury site. One year postoperative radiographic and histologic observations showed complete regeneration of bone at the injury site with a defect-fill of atleast 1.5 cm height[6].

g. Pradel and Lauer compared bone formation in eight children with complete cleft lip, alveolus and palate using two methods. Four children received autologous bone grafts from iliac bone and the other four received tissue engineered bone (autogenous osteoblasts

harvested from maxillary bone biopsy cultured on demineralized bone matrix). Results showed a higher percentage of defect ossification in the patients who received tissue engineered bone. They concluded that tissue engineered bone is a promising alternative in alveolar bone grafting with no disadvantages[7].

b) Significance of study

There are many sources of osteogenic precursor cells in the body i.e. marrow, periosteum, adipose, umbilical, dental pulp etc., but the risk of donor site morbidity and the difficulty in acquiring adequate volumes of tissue to isolate stem cells remain hurdles for clinical implementation. Since the use of progenitor cells for regenerative medicine requires the harvesting of healthy cells from the patient prior to the main surgery, priority should be placed on minimization of patient pain and trauma while ensuring adequate cell sourcing. The significance of the present study would be:

1. Identification of ideal source of osteogenic precursor cells for bone tissue engineering
2. This study will form a basis for clinical application of isolation and culture of osteogenic precursor cells for bone tissue engineering
3. This study will benefit numerous patients who require bone reconstruction especially in areas where bone formation does not occur physiologically
4. Study will benefit students of dental science and biotechnology in gaining knowledge and skills with interdisciplinary approach
5. Study will serve as preliminary work towards establishment of bone tissue engineering laboratory and learning center
6. A pilot study for future randomized controlled clinical trials in the field

c) Its potential contribution to knowledge in the field of social relevance/ national importance

Social Relevance - The research project on completion, will add to the existing knowledge in treatment of numerous patients using the proposed source of cells for bone regeneration in areas where bone formation does not occur naturally. This will help immensely in improving the quality of life of

patients. The knowledge and skills gained will enhance student education in the innovative field of tissue engineering.

National importance - There is no published material available on the comparison of sources for osteogenic precursor cells for tissue engineered bone in India. This would be the first of its kind. The knowledge gained will be instrumental in setting up a bone tissue engineering laboratory and learning center, the first of its kind in India.

Objectives:

1. Isolation and culture of osteogenic precursor cells from the three sources in the oral cavity i.e. periosteum, cancellous bone and dental pulp
2. Comparison of cultured osteogenic precursor cells obtained from the three sources based on -
    a. Cell proliferation activity
    b. Alkaline phosphatase activity
    c. Mineralization activity

Methodology

Study type – Invitro comparative study

Study site – Faculty of Dentistry (Sample collection) and Dept. of Biosciences (Sample processing, Cell isolation and culture, Cell Analysis), Jamia Millia Islamia

Precautions in isolation and culture of cells – All biopsies will be done with aseptic precautions as prescribed by ICMR. Biopsies will be stored and transported in Dulbecco's modified Eagle's medium high glucose (DMEM-H). All biopsies will be processed for cell isolation within 2 hours of tissue harvesting to maintain adequate cell viability[8]. All procedures will be as per the National Guidelines for Stem Cell Research published jointly by ICMR and DBT in 2017.

1. Isolation and culture of cells from human periosteum – 10 Human periosteum biopsies will be harvested from patients during the course of oral surgery (wisdom tooth removal). The cells will be cultivated using an osteogenic medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU penicillin/ml, 100ug/mL streptomycin and 1mmol/L ascorbic acid at 37^0C with 5% CO_2. Cells will be seeded after the second passage. During passaging, cells will be detached from 75cm^2 cell culture flasks using 5ml of a 0.05% trypsin solution in phosphate-buffered saline (PBS). After a 1:1 dilution of the cell suspension with DMEM containing 10% FCS and centrifugation at 3200g for 3min, cells will be resuspended in DMEM containing 10% FCS, counted and reseeded at a density of 10^5cells per 75cm^2 cell culture flask. Cells will be cultured in the same medium used for cell seeding in a humidified atmosphere with 5% CO2 at 37^0C[9].
2. Isolation and culture of cells from human cancellous bone – 10 cancellous bone biopies will be taken from the mandible during the course of oral surgery (wisdom tooth removal). Biopsies will be rinsed in 70% ethanol and three times in phosphate buffered saline. Samples will be separated into pieces of 2x1mm size and cultured as explants in 25 cm^2 flasks in a humidified atmosphere of 95% air and 5% CO_2 in a growth medium containing DMEM, 20% FBS, 1%

nonessential amino acid, 100ng/ml sodium pyruvate, 100 IU penicillin, 100ug/mL streptomycin at 37^0C. The cells will be subcultured twice after 10 days[10].

3. Isolation and culture of cells from dental pulp tissue – Pulp tissue will be harvested from 10 non-carious teeth of patients undergoing extraction for therapeutic reasons (Orthodontic extractions). The extracted teeth will be washed in 0.2% chlorhexidine solution; the pulp chamber opened using a drill and pulp collected. The pulp will be rinsed in 1.5ml saline solution and mechanically dissociated. Cells will be filtered and cultured in DMEM, 20% FBS and the medium will be changed twice a week. At day 21, cells will be detached using 0.02% EDTA solution, centrifuged and incubated in 100 ml PBS solution for 1 hour at 4^0C.
4. Analysis of osteogenic precursor cells – this will be done using the following three tests –
    a. Assessment of cell proliferation

        Cells obtained from the three sources will be seeded in 96-well plates. Cell proliferation analysis will be performed using the MTT assay. The assay is based on the ability of mitochondrial dehydrogenase to oxidase thiazolyl blue to an insoluble blue formazan product. The optical density of the plates will be read on microplace reader using test and reference wavelength of 540 and 620 nm, respectively[1]. The growth curves of each source will be drawn and the absorbance will be compared statistically.

    b. Assessment of activity of alkaline phosphatase (ALP)

        Cells obtained from the three sources will be seeded in 24-well plates and rinsed twice with PBS followed by trypsinization and then scraped into ddH_2O. This will be followed by three cycles of freezing and thawing. ALP activity will be determined at 450 nm using p-nitrophenyl phosphate (pNPP) as the substrate. 50 uL of the sample will be mixed with 50 uL pNPP (1mg/ml) in 1 M diethanolamine buffer containing 0.5 mM $MgCl_2$, pH 9.8 and incubated at 37^0C for 15 min on a bench shaker. Next, 25 uL of 3 M NaOH/100 uL will be added to stop the reaction.

Enzyme activity will be quantified by absorbance measurements at 405 nm. Total protein content will be determined with the BCA method and read at 562 nm[3].

c. Mineralization assays

Cells will be seeded in 24-well plates. Plates will be washed three times with PBS and then stained with alizarin red S in H_2O, pH 4.0 for 1 hour at room temperature. After staining, cultures will be washed three times with H_2O followed by 70% ethanol. Cultures will then be incubated in 100 nm cetylpyridinium chloride for 1 hour to solubilize and release calcium-bound alizarin red S in to solution. The absorbance of the released alizarin red S will be measured at 562 nm[4].

5. Analysis of results – All data will be analysed using SPSS software. Statistical analysis of the data will be performed by one-way analysis of variance (ANOVA) and post hoc test for multiple comparisons will be done using Fisher LSD test. For non-homogenous variance, Kruskal-Wallis test will be used[1].

Year wise plan of targets

	0-8 months	8-12 months	12-20 months	21-32 months	33-36 months
Procurement of reagents and chemicals					
Trial run of Laboratory and chemicals					
Harvesting of source tissue from patients					
Isolation and culture of desired cells					
Qualitative analysis of cells					
Analysis, documentation and report					

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